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Evaluation by Polymerase Chain Reaction on the Effect of Betapropiolactone and Binary Ethyleneimine on DNA

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Abstract. Inactivating treatments for viruses such as pasteurization or alkylation by β-propiolactone or binary ethyleneimine were tested for their capacity to modify nucleic acids. The modification of a nucleic acid was measured as the decrease in spot intensity in Southern blots after polymerase chain reaction (PCR) amplification. The inactivating treatments were applied to cellular and viral genomic material from a human lymphoblastoïd cell line immortalized by Epstein Barr Virus (EBV), which produced a monoclonal antibody.

Pasteurization did not modify the ability to amplify and detect cellular or viral DNA. Binary ethyleneimine strongly reduced the amount of detectable DNA and β-propiolactone under particular conditions of incubation abolished all trace of DNA.

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Key Words: DNA inactivation; PCR; β-propiolactone; Binary ethyleneimine.

Introduction

If a continuous cell line is to be used to produce a monoclonal antibody for therapeutic purposes the final product must be demonstrated to be free from viral contamination when the cell line is known to carry an infectious virus. Moreover the cellular DNA content has to be tested to verify that the amount in residual cellular DNA in the final product has been reduced to an acceptable level. The aim of these investigations is to ensure the absence of undesired biological activity such as oncogenicity or infectivity.[1] The methods used to detect these kinds of biological activities are time consuming and expensive, because they require long-term observation of animals and/or cells inoculated with the therapeutic product.

Potentially, tests for these biological activities can be avoided if the absence of genomic material can be clearly demonstrated, as infectivity is associated with viral genomes and oncogenicity with cellular DNA. The polymerase chain reaction (PCR) can detect as little as one single molecule of DNA and is therefore suitable for screening for traces of genomic material.

β-propiolactone (BPL)[2] and binary ethylenimine (BEI)[3] are known to alkylate nucleic acids. We investigated various conditions of alkylation which results in genomic DNA becoming undetectable even after amplification by PCR of DNA from a cell line deliberately contaminated with Epstein Barr Virus (EBV). Alkylation of DNA implies that the nucleic acid was modified and its modification was measured as the decrease in spot intensity obtained after Southern blot with a probe which hybridizes the unmodified DNA.

Materials and methods

The F5 cell line

A human B lymphocyte was immortalized by EBV infection to produce a monoclonal antibody specific to Rh(D) blood group antigen.[4] The cells were grown in a hollow-fiber cartridge in serum free medium[5] and secreted the immunoglobulin in the supernatant.

Preparation of DNAs

High molecular weight DNA from peripheral blood leucocytes (PBL) of one healthy donor or from the lymphoblastoïd cell line was purified according to Blin *et al.*[6] Briefly, the cells were incubated overnight at

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56°C under agitation, in 100 mM Tris, 10 mM EDTA pH 7·5, containing SDS (1%) and proteinase K (0·2 mg/ml) (Boehringer, Mannheim, GmbH). The DNA was extracted with phenol: chloroform (1:1), and then chloroform:isoamylalcohol (24:1). The nucleic acids in the aqueous phase were precipitated with ethanol. The DNA was resuspended in 10 mM Tris-HCl, 1 mM EDTA pH 8·0, its concentration determined by measuring UV absorbance at 260 nm and adjusted to 1 mg/ml.

The BamC region of the EBV-DNA was inserted into a plasmid (pBR 322) and used as a positive PCR control.

Non-specific control DNA was from *S. cerevisiae* (Clontech, Ozyme, France) and the size markers were Lambda DNA and pBR 322 (Bethesda Research Laboratories, Gaithesburg, MD). DNA was digested according to the recommendations of the restriction enzyme supplier (Boehringer, Mannheim, GmbH).

Oligonucleotide primers and probes

Two 20 mer oligonucleotides primers (PG244:5′ GAC AAC TCG GCC GTG ATG GA 3′ and PG245:5′ TGA AGT TGG AGG CGG ACG AG 3′) corresponded to sequences of 120 bp fragment in the BamC region of EBV-DNA[7] and the 38 mer probe (PG246:5′ TGG CCT GGG CGT GAA GCT GAC CTT TGG CTC GGC CTC CT 3′) corresponded to a sequence in the central region of the PCR-amplified product.

Primers and probe (PC03, PC04 and RS06) corresponding to a 110 bp region of the β-globin gene[8] were also used. The oligonucleotides were synthesized in a Millipore model 8700 automated DNA synthesizer by the betacyanoethyl-protected phosphoramidite technique.[9]

PCR amplification

DNA was denatured at 95°C for 10 min and cooled rapidly on ice before amplification according to the methodology of Saiki.[10,11] The AmpliTaq recombinant Taq DNA polymerase (2·5 units) from Perkin Elmer-Cetus (Norwalk, CT) was added to 100 μl of DNA solution. The solution contained 100 μmoles of each primer and 20 μmoles of each d NTPs (Pharmacia, Uppsula, Sweden), in a 10 mM Tris-HCl buffer pH 8·3 with 50 mM KCl and 0·01% gelatin (Biorad, Richmond, CA, U.S.A.) The amplification needed 1·5 mM $MgCl_2$ for the EBV-DNA samples, and 0·75 mM for the β-globin samples. The reaction mixture was overlaid with 50 μl of mineral oil (Sigma, St Louis, MO, U.S.A.) to prevent condensation. Amplification (30 or 45 cycles) was performed in a thermocycler PH-C2 (Techne, France). Each cycle included denaturation (95°C for 1 min), annealing (54°C for 0·5 min) and elongation (72°C for 2 min). For the last cycle, elongation lasted 7 min. The amplification products were kept at 4°C.

Southern blot analysis

Amplification products (10–30 μl) were electrophoresed on a 4% agarose gel (3% Nu-Sieve GTG, 1% Seakem, FMC Bioproducts, Rockland, ME, U.S.A.) in Tris-borate EDTA buffer. DNA was stained with ethidium bromide, transferred to a Nytran-N nylon membrane (Schleicher and Schuell, Keene, NH, U.S.A.)[12] and exposed to UV for 2 min. The filters were prehybridized for 2 h at 42°C in prehybridization buffer containing formamide (50%), 5 × SSC buffer, 5 × Denhardt's solution, SDS (1%), EDTA (1 mM), Tris HCl (20 mM) pH 7·5, and denatured sonicated salmon-sperm DNA (100 μg/ml). The filters were then probed for hybridization overnight at 42°C with 10^6 cpm/ml of ^{32}P radiolabeled probe in the same solution supplemented with 10% dextran sulfate. Filters were then washed three times in 2 × SSC, 0·1% SDS for 15 min at room temperature and once in 0·1 × SSC, 0·1% SDS for 15 min at 70°C. Films were exposed to the filters for 6 h or 24 h with intensifying screens at −80°C.

Slot-blot analysis

Samples (10 μl) were applied to a Nytran-N nylon membrane on a Minifold-II apparatus (Schleicher and Schuell) and then, the membranes were hybridized as described above.

DNA radiolabeling

The synthetic probes were end-labeled with (γ-^{32}P)dATP 5000 Ci/mmole, Amersham, France) by incubation with T4 polynucleotide kinase[13] from Boehringer Mannheim (Meylan, France). High molecular weight DNA was labeled with (α-^{32}P)dCTP (3000 Ci/mmole, Amersham, France) by nick-translation and the incorporation was stopped by TCA precipitation.[12]

Treatment with chemicals

β-propiolactone (BPL) was obtained from Serva (St Germain en Laye, France) and stored at −20°C. A solution of 1% BPL in 15 mM KH_2PO_4 pH 8·0 was freshly prepared for each experiment. BPL was added to DNA solutions to give final concentrations of 0·025%, 0·05 % and 0·1%. The samples were incubated either at 4°C or at 37°C in buffers of pH range 6·5 to 8·0.

Binary ethyleimine (BEI) was prepared by cyclization of 0·1 M 2-bromoethylamine-HBr in 0·2M NaOH at 37°C for 1 h. The β-naphthol violet (a pH indicator) was added to the solution to check the formation of BEI which causes a change in color from violet to orange. This solution was added to DNA samples to a concentration of 1% (v/v). The solutions were incubated either at 25°C for 17 h or 37°C for 5 h and the reaction was stopped by addition of sodium thiosulfate.[14,15]

Pasteurization

Immunoglobulin secreted by the F5-cell line was purified from culture supernatant. Purified immunoglobulin preparations spiked with the plasmid or F5-DNA were incubated at 60°C for 10 h.

Results

Cellular DNA is damaged by BPL

The effects of pH. The modification of cellular F5-DNA incubated with BPL at 4°C during 72 h was influenced by the pH (Fig. 1b). DNA incubated without BPL was successfully amplified by PCR whatever the pH. The most intense signals were obtained at pH 7·4 or 8·0. When the samples were incubated with BPL, the most intense amplification signal was similar to that of the control sample containing 1 pg prior to PCR (b19). However the treated sample contained 1 μg of DNA and thus there was a 6 log decrease in amplifiable DNA. This loss of signal was observed for all samples incubated at pH 7·4 and 8·0, even at the lowest concentrations of BPL (b3 and b4).

Effect of incubation time. F5-DNA was incubated at 4°C in buffer pH 8·0, containing a low (0·025%) or a high (0·1%) concentration of BPL. Samples were taken at 16 h, 48 h, and 96 h for PCR amplification (Fig. 2b and d). The product amplified from the 0·025% BPL sample was clearly visible after 16 h of incubation and weakly visible after 48 h; only a minute amount of product was detected by ethidium bromide staining after 96 h. Southern blot analysis gave similar results (Fig. 2d).

Alkylation was much faster with an initial concentration of 0·1% of BPL. Ethidium bromide staining did not reveal any bands from the amplification products (Fig 2, b8) and only traces of product were detected by hybridization after 48 h of incubation (d8), and no signal was detected after 96 h (b9 and d9).

Therefore the higher the concentration in BPL, the faster the DNA inactivation. Furthermore, unhydrolized BPL remained active in aqueous solution

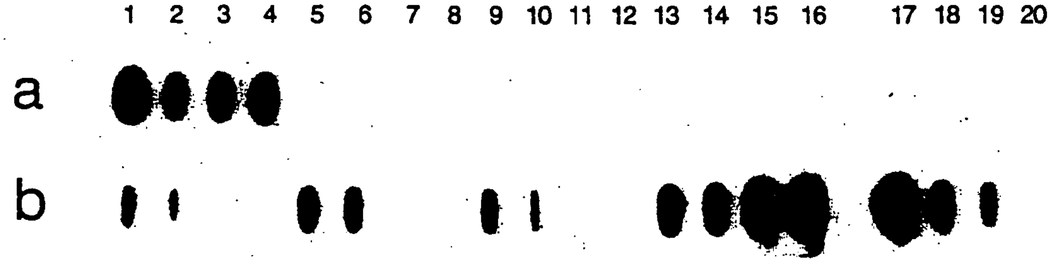

Figure 1. Detection of EBV-sequence incubated with BPL at various pH and temperatures. PCR-amplified DNA products were analyzed by slot blotting.

(a) Plasmid-DNA (1 μg) was incubated with 0·1% pH 8·0 either at 4°C for 40 h (a2) either at 37°C for 2 h (a3); (a1) and (a4) were the respective control samples incubated without BPL.

(b) F5-DNA (1 μg) was incubated at 4°C for 72 h with various concentrations of BPL in phosphate buffer of pH range 6·5 to 8·0:

	BPL (%)			
pH	0	0·025	0·05	0·1
6·5	b13	b1	b5	b9
7·0	b14	b2	b6	b10
7·4	b15	b3	b7	b11
8·0	b16	b4	b8	b12

The slots b17, b18, b19 and b20 were the control samples containing respectively 1 μg, 1 ng, 1 pg and no DNA.

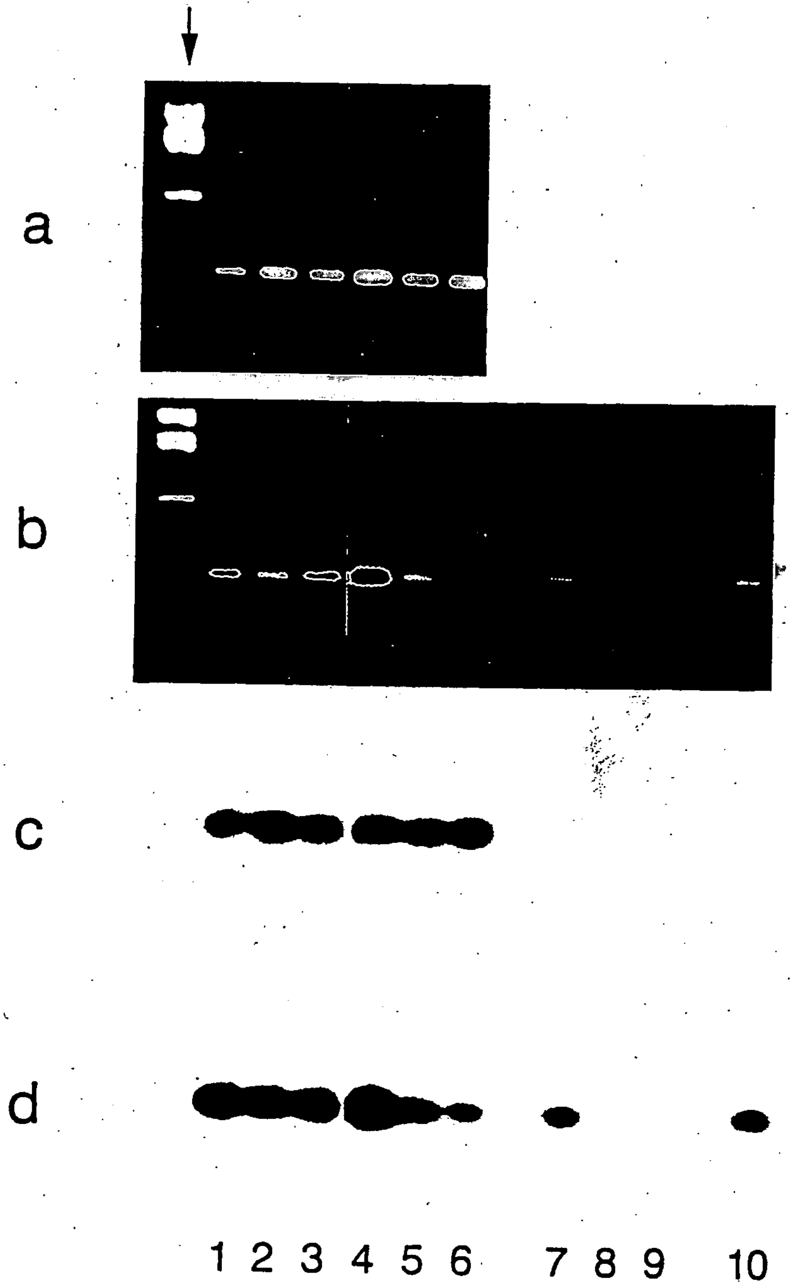

Figure 2. Effect of the time of incubation with BPL. PCR-amplified DNA products were electrophoresed on agarose gel and analyzed by Southern blotting with EBV probe. The arrow indicates the Bst NI digest fragments from pBR 322.

(a) Plasmid-DNA (1 μg) was amplified either alone (a4, a5 and a6) or with 0·1% BPL treated DNA (0·1 μg) from *S. cerevisiae* (a1) or from the plasmid itself (a2). Samples containing BPL were incubated at 4°C for 96 h, pH 8·0. The concentrations of added BPL were respectively 0·025 and 0·1% for the samples a4 and a5; the sample a6 was incubated without BPL. The sample a3 was a positive PCR control.

(b) F5-DNA (1 μg) was amplified either alone (b4 to b10) or with 0·1% BPL treated DNA (0·1 μg) from *S. cerevisiae* (b1) or from F5 cells (b2). Samples containing BPL were incubated at 4°C, pH 8·0 for various times of incubation:

	0·025% BPL	0·1% BPL
16 h	b4	b7
48 h	b5	b8
96 h	b6	b9

The sample b10 was incubated without BPL and the sample b3 was a positive PCR control.
(c) and (d): autoradiographs of the Southern-blot analysis of a and b respectively.

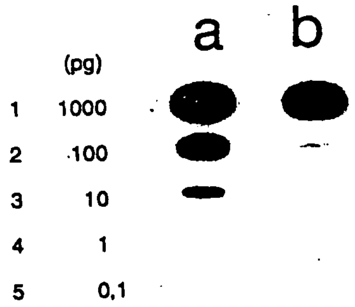

Figure 3. Influence of the DNA content. Plasmid-DNA was PCR-amplified and one tenth volume was spotted; the corresponding quantities prior to PCR are indicated, ranging from 1000 to 0·1 pg. (a) control DNA, incubated without BPL; (b) DNA incubated with 0·1% BPL, pH 8·0 for 72 h at 4°C.

after 48 h at 4°C since some DNA was modified between 48 and 96 h of incubation.

Efficiency of the BPL depends on the DNA content

The plasmid was treated with BPL at the pH and temperature which were as the most effective for alkylation of F5-DNA. The difference between BPL (0·025% and 0·1%) treated and untreated samples (Fig. 2, a4, a5 and a6) at 4°C for 96 h was small when the DNA concentration was 10 μg/ml. Alkylation by 0·1% BPL was not modified by increasing the incubation temperature to 37°C (Fig. 1, a3).

Various lower concentrations of plasmid DNA(100 ng to 10 pg per ml) were incubated with 0·1% BPL at pH 8·0 for 72 h at 4°C. After incubation, the target DNA sequence for EBV was amplified by PCR and the products analyzed by slotblot (Fig. 3). Amplified products were detected in the untreated samples containing 100, 10, 1 ng DNA per ml (a1, a2, and a3) and non detected in the samples containing 100 pg and 10 pg DNA per ml (a4 and a5). No amplified product was detected in the BPL treated samples containing 1 ng DNA (b3) or less per ml (b4 and b5). Treated and untreated samples were compared by densitometric scanning: inhibition of amplification was 40% at 100 ng DNA per ml (b1) and more than 90% at 10 ng DNA per ml (b2).

BPL does not inhibit the polymerase chain reaction

To ensure that the yield of PCR amplification was not affected by residual BPL or its degradation products (e.g. β-hydroxy-propionic acid) or contaminants in the DNA preparations, control DNA was supplemented with one-tenth volume of specific or non specific DNA solution pretreated with 0·1% BPL at pH 8·0 and 4°C for 96 h. This mixture was amplified by PCR. In all cases, DNA amplification was similar as assessed by ethidium bromide staining (Fig. 2, a1 and a2, b1 and b2) and blotting (Fig. 2, c1 and c2, d1 and d2).

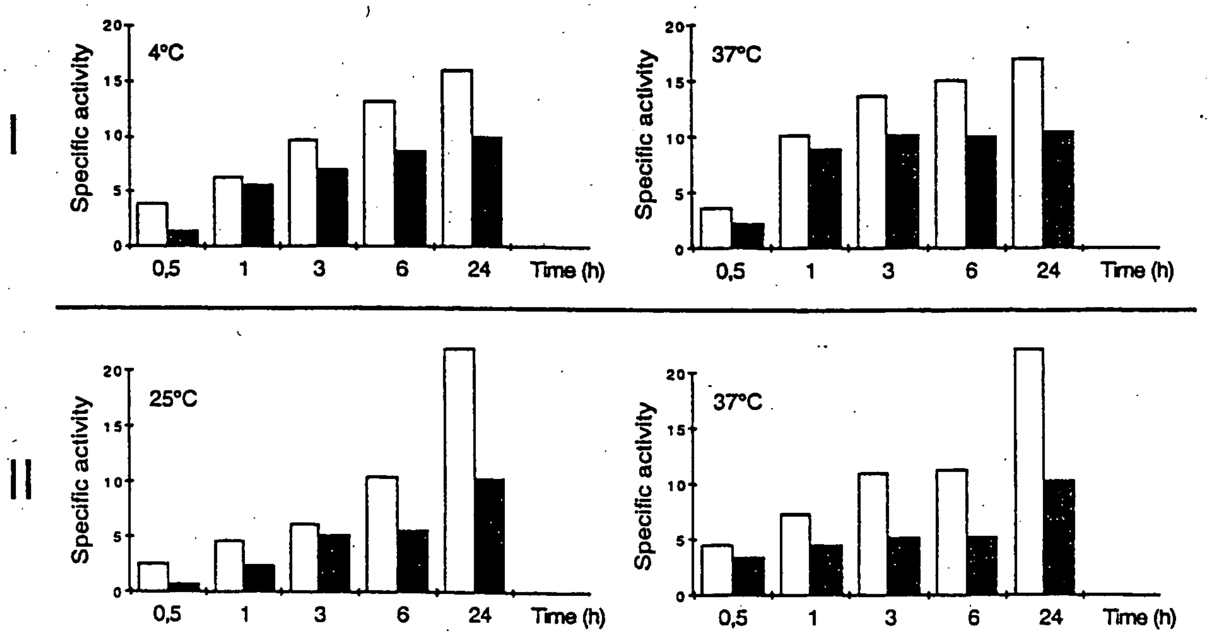

Figure 4. Kinetics of DNA synthesis treated with alkylating agents. (I) PBL-DNA (1 μg) was incubated with 0·1% BPL, pH 8·0 either at 4°C or 37°C (grey bar). (II) PBL-DNA (1 μg) was incubated with 1% BEI either at 25°C or at 37°C. Specific activity is expressed as 10^7 cpm/μg and white bars represent control DNA.

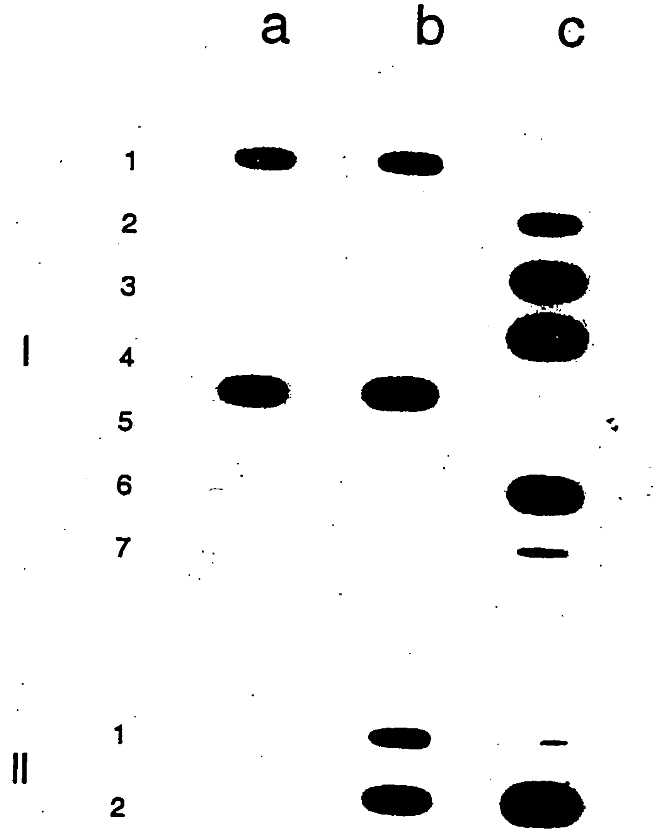

Figure 5. Detection of EBV and cellular sequences after alkylation by BPL or BEI. PCR-amplified DNA products were analyzed by Slot blotting.

(I) Slot blot with EBV probe. (a) PBL-DNA (1 μg) incubated with 0·1% BPL pH 8·0 at 4°C for 72 h (a1), a control sample was incubated without BPL (a5); (b) PBL-DNA (1 μg) incubated with 1% BEI at 37°C for 5 h (b1), a control sample was incubated without BPL (b5); (c) Plasmid-DNA; amplified 1 μg (c6) and 1 pg (c7); non-amplified 100 ng (c2), 500 ng (c3), 1000 ng (c4).

(II) Slot blot with β-globin probe. (b) PBL-DNA (1 μg) incubated with 0·1% BPL pH 8·0 at 4°C for 72 h (b1), a control sample was incubated without BPL (b2); (c) PBL-DNA (1 μg) incubated with 1% BEI at 37°C for 5 h (c1), a control sample was incubated without BPL (c2).

Comparing BEI with BPL

PBL-DNA was incubated with either 0·1% BPL pH 8·0 (Fig. 4I) or 1% BEI (Fig. 4II) and the samples were nick-translated to incorporate α P^{32} dCTP. The rate of incorporation dropped to 40% and 50% of the control values in the samples treated with BPL and BEI respectively. At all temperatures of incubation, the same final result was obtained. However, the incorporation was low in the sample incubated at 4°C with BPL between 0·5 and 24 h, whereas incorporation after 1 h was not more than after 24 h in the DNA treated with BPL at 37°C. In the first 6 h of incubation at 37°C, the DNA treated with BEI incorporated less nucleotide than DNA treated with BPL and the final level of incorporation was identical.

Target DNA sequences in EBV and the β-globin gene were amplified by PCR from PBL-DNA (Fig. 5I and 5II respectively) and the products were analyzed by slot-blotting. About a 50% reduction in spot intensity was observed after hybridization with the EBV probe (a1 and b1) whatever the chemical used in comparison with the non treated sample. The β-globin probe gave a stronger hybridization signal with the BPL treated DNA than with the BEI treated DNA (b1 and c1).

Pasteurization does not modify the DNA detection

The effect of heating was investigated using the purified immunoglobulin preparation deliberately contaminated with F5-DNA or plasmid. The EBV-sequence was amplified by PCR and analyzed by Southern blotting (Fig. 6). For both plasmid and F5-DNA, the intensity of the signal observed on the Southern blot was not modified when the sample was heated to 60°C for 10 h[4,5] or kept at 4°C.[2,3] The spot intensities were similar to those of the positive PCR controls.[6,7] Therefore heat did not appear to damage the DNA.

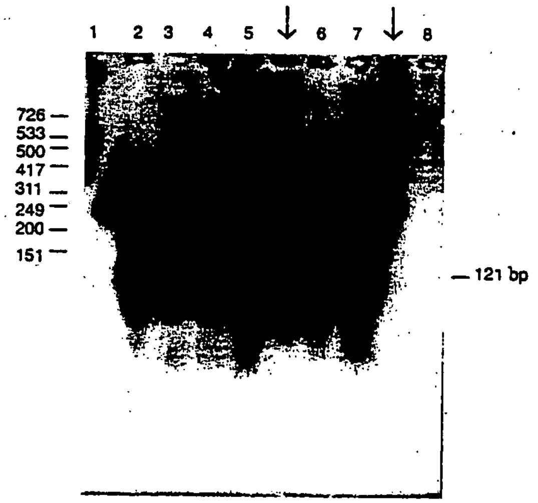

Figure 6. Detection of EBV sequences in pasteurized samples. A purified preparation of immunoglobulin (IgG), spiked with 6 μg of plasmid or F5-DNA was incubated for 10 h at 60°C (lanes 4 and 5 respectively). Control samples, unspiked IgG (lane) and FS-DNA (lane 2) or IgG spiked with F5-DNA (lane 3) were kept at 4°C for the same time. F5 and plasmid-DNA were amplified as PCR positive controls (lanes 6 and 7 respectively). Lane 8 was the reagent control and lanes under arrows contained size markers (lambda HinfIII and pBR 322 BstNI).

Discussion

A low temperature (4°C) was sufficient to modify cellular DNA or plasmid DNA in presence of 0·1% BPL. BPL treatment at 4°C is the most widely used method to inactivate a wide range of viruses,[16] particularly the rabies virus for the production of human vaccines.[17] This treatment was shown to impair the biological activity of cellular DNA.[23] Generally, inactivation with BPL is achieved by heating the virus suspension at 37°C to hydrolyze the residual inactivating agent. This is justified as the modification observed between 48 h and 96 h of treatment suggests that active chemical is still present in solution after two days at 4°C. Indeed, undamaged DNA was detectable at 48 h but not after 96 h. The rate of nucleotide incorporation by nick translation was identical for BPL treatment at 37°C and 4°C. Moreover, the chemical was not active after 1 h at 37°C, probably as a consequence of hydrolysis of the lactone. Heating to 37°C alone did not modify the detectability of the DNA after 2 h of incubation. Therefore the decrease of signal observed is only ascribable to a chemical reaction with BPL.

The reactivity of BPL with DNA is extremely sensitive to pH. The efficacy of the reaction was much greater at weakly basic pH (7·4 and 8·0) than at neutral pH (7·0) or weakly acid pH (6·5). It may thus be possible to use lower concentrations of BPL for complete inactivation in a medium at pH 8·0. Unfortunately there are little data in the literature about the influence of pH variation on the inactivation kinetics of RNA or DNA viruses. Generally the pH range is 7·0 to 7·5 when inactivating RNA viruses like poliovirus[18] or HIV[19] with BPL.

Binary ethyleneimine (BEI) is also widely used (for a review see ref. 14) and has been used in a standard protocol to inactivate some viruses so as to manufacture veterinary vaccines, like the FMDV vaccine. The damage caused to the DNA was revealed by the reduced incorporation by nick translation of nucleotides in the BEI-treated sample, and also by hybridization of cellular DNA with the β-globin probe. The hybridization experiments suggest that the damage to the nucleotides are more severe in the BEI than in the BPL treated DNA.

Other factors influencing the ability of a chemical to damage DNA could be the frequency of the target nucleotide (mainly guanin) for BPL in the DNA molecule. Obviously number of targets increases with the size of the genome.

Cellular F5-DNA was more sensitive to BPL treatment than the DNA of peripheral blood leucocytes. This preparation of DNA from PBL of a healthy donor was found positive in EBV genome. This is in accordance with the fact that more than 90% of the population is asymptomatically infected.[20] Inactive and latent infection of the peripheral blood lymphocytes may differentiate these polyclonal cells from F5 cells. This F5 cell line was deliberately contaminated with EBV to immortalize it and the cells continuously secrete infectious virus (results not shown) revealing the persistance of the full virus genome.

EBV is a lymphotropic herpes virus with a double stranded genome of 172 kbp.[21] This corresponds to a large target to an alkylating agent. Conversely, the BamC fragment of the EBV-DNA used in this study was 8 kbp long and therefore a 22 times smaller target than the full viral genome. Our preliminary results show that the alkylating agents, BPL and BEI, efficiently damage DNA. The effectiveness of the alkylation has to be considered not only for DNA but also for RNA. In veterinary medicine BEI is the preferred inactivating agent for the vaccines containing animal viruses with DNA or RNA genomes. In particular, it is preferred to formaldehyde for its more complete and rapid inactivation of Foot and Mouth Disease Virus.[22] However, it has never been used in human medicine.

We show that BPL under particular conditions destroys the template function of DNA since PCR failed to generate a product capable of hybridizing a probe specific of the unmodified DNA. In these conditions, PCR could be also a predictive *in vitro* method for the absence of biological activity linked to a genome.

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